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An efficient and flexible system for construction of adenovirus vectors with insertions or deletions in early regions 1 and 3

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ABSTRACT **Human adenoviruses (Ads) are attracting considerable attention because of their potential utility for gene transfer and gene therapy, for development of live viral vectored vaccines, and for protein expression in mammalian cells. Engineering Ad vectors for these applications requires a variety of reagents in the form of Ads and bacterial plasmids containing viral DNA sequences and requires different strategies for construction of vectors for different purposes. To simplify Ad vector construction and develop a procedure with maximum flexibility, efficiency, and cloning capacity, we have developed a vector system based on use of Ad5 DNA sequences cloned in bacterial plasmids. Expanded deletions in early region 1 (3180 bp) and early region 3 (2690 or 3132 bp) can be combined in a single vector that should have a capacity for inserts of up to 8.3 kb, enough to accommodate the majority of cDNAs encoding proteins with regulatory elements. Genes can be inserted into either early region 1 or 3 or both and mutations or deletions can be readily introduced elsewhere in the viral genome. To illustrate the flexibility of the system, we have introduced a wild-type early region 3 into the vectors, and to illustrate the high capacity for inserts, we have isolated a vector with two genes totaling 7.8 kb.**

Construction of adenovirus (Ad) vectors involves insertion of foreign DNA into the Ad genome, usually with compensating deletions in early region 1 (E1) or early region 3 (E3). E1 is not required for viral replication in 293 cells (1), which express the left 11% of the Ad5 genome. For viral viability, deletions in this region must not affect the inverted terminal repeat (ITR; 1–103 bp) or packaging signals (194–358 bp) (2–5). In addition, deletions should not extend into the coding sequences for protein IX, which is essential for packaging of full-length genomes into functional virions (6). Since Ad virions can package ≈105% of the wild-type (wt) genome length (7), deletions of up to 2.9 kb in E1 (8–10) permit construction of defective vectors with inserts up to 4.7–4.9 kb.

E3 is nonessential for viral replication in any normally permissive human cell and can be deleted to produce nondefective vectors (11, 12). Deletions in E3 presumably cannot extend into essential virion structural genes, pVIII and fiber, flanking this region. Several E3 deletions have been used for vector construction, the most common resulting from removal of 1.88 kb of E3 sequences between *Xba* I sites at 79.6 and 84.8 map units (mu) in the Ad5 genome (11, 12), providing a capacity for inserts of 3.7–3.9 kb.

Among current methods for generating Ad vectors (8), there is no simple procedure for generating vectors with both E1 and E3 deletions. To construct Ad5 vectors that combine E1 and E3 deletions or substitutions and to simplify Ad vector production, we have developed a methodology based on a series of bacterial plasmids (pBHG) containing most of the viral genome in circular form but lacking the DNA packaging signals. In this paper, we describe this system and its use to generate vectors with a wt E3 region or with inserts of up to 7.8 kb of foreign DNA in the E1 region.

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MATERIALS AND METHODS

Construction of Recombinant Plasmids. Plasmids constructed by standard protocols (13) were used to transform *Escherichia coli* DH5 (13) by electroporation (14) or *E. coli* HMS174 by using $CaCl_2$. Plasmid DNA was prepared by the alkaline lysis method (15) and purified by CsCl–ethidium bromide density gradient centrifugation.

Cells and Viruses. Cell culture media were obtained from GIBCO. Ad vectors were propagated and titered on 293 cells as described (16). Recombinant viruses were isolated by cotransfection of 293 cells with appropriate plasmids or viral DNA (17). After 8–10 days, plaques were isolated and expanded, and viral DNA was analyzed by restriction enzyme digestion as described (12, 16). [^{35}S]Methionine labeling, immunoprecipitation, and SDS/PAGE were carried out as described (16, 18). Densitometry was performed using the LKB Ultroscan XL enhanced laser densitometer.

RESULTS

Generation of the Plasmid pBHG10. In developing the strategy to be described, advantage was taken of previous observations made by ourselves (10, 12, 19–21) and other investigators (2, 11, 22–24). One key finding is the fact that Ad DNA can circularize in infected cells (19) and that this phenomenon can be exploited to generate infectious circular Ad genomes that can be propagated as bacterial plasmids (10, 20, 21). Secondly, it has been shown that the cotransfection into mammalian cells of two plasmids with overlapping sequences can generate infectious virus with good efficiency (11, 12, 21). The third finding important to this strategy is that Ads carry a cis-acting sequence in the left end of the genome that is essential for encapsidation of viral DNA (2, 22–24). When this cis-acting signal, located from bp 194 to 358 in Ad5, is deleted, viral genomes cannot be packaged but are expected to replicate their DNA in transfected cells (2–5).

These findings led us to design and execute the strategy outlined in Fig. 1. The first step involved the construction of AdBHG, a virus that contains the Ad5 genome with the deletion of E3 sequences from bp 28,133 to 30,818 and the insertion of modified pBR322 at bp 1339. AdBHG was made by cotransfection of 293 cells with purified viral DNA from Ad5PacI, digested with *Cla* I and *Xba* I, and pWH3.

The next step involved the generation of a bacterial plasmid containing the entire AdBHG genome and subsequent identification of infectious clones. Baby rat kidney (BRK) cells were infected with AdBHG under conditions that result in the generation of circular Ad5 genomes (10, 19). At 48 h after infection DNA was extracted from the infected BRK

Abbreviations: Ad, adenovirus; E1 and E3, early regions 1 and 3, respectively; wt, wild type; ITR, inverted terminal repeat; mu, map unit(s); Ap^r, ampicillin resistance; Tet^r, tetracycline resistance; Kn^r, kanamycin resistance; HCMV, human cytomegalovirus; HSV-1, herpes simplex virus type 1.

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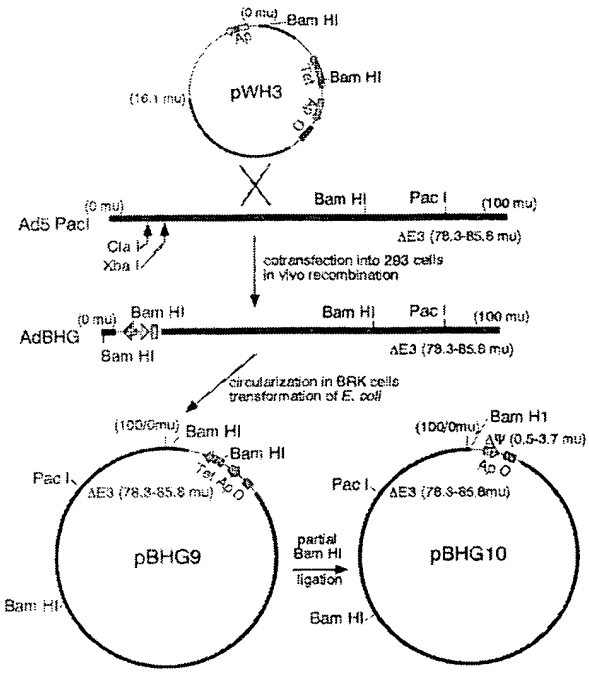

FIG. 1. Construction of pBHG10. pWH1 (not shown) was constructed from the plasmid pKH188 (25, 26) (derived by insertional mutagenesis of the E1A region in pKH101 resulting in the introduction of a *Bam*HI site at bp 188 in the Ad5 genome) by inserting pBRX (27) (a pBR322 derivative with an *Xba* I site at nt 2066) into the *Xba* I site at bp 1339 in Ad5 sequences. pWH1 was then combined with pXC38 (25) to include Ad5 sequences from 5.7 to 16.1 mu, generating pWH3. Ad5PacI [derived by cotransfection of 293 cells with pFG173 (18) and pAB14PacI, a modification of pAB14 (7) that substitutes a *Pac* I cloning site for 2.69 kb of E3] was digested with *Cla* I and *Xba* I and cotransfected into 293 cells with pWH3 to generate AdBHG. In the next step, the AdBHG genome was circularized by infecting BRK cells at multiplicity of infection of ≈20 under conditions that result in the generation of circular Ad5 genomes (10, 19). At 48 h after infection, DNA was extracted from the infected BRK cells and used to transform *E. coli* HMS174 to Ap^r and Tet^r. Small-scale plasmid preparations were made from the colonies obtained and screened by *Hin*dIII and *Bam*HI/*Sma* I digestion followed by gel electrophoresis (data not shown). Four candidates that appeared to possess a full AdBHG genome with intact junction regions were tested for infectivity and sequenced in the region of the junction. A single infectious clone was chosen, pBHG9. In the final step, the packaging signals were deleted from pBHG9 by partial *Bam*HI digestion and religation generating pBHG10. mu refers to Ad5 sequences, solid bars represent Ad5 sequences, and hatched bars represent Ap^r, Kn^r, and Tet^r segments.

cells and used to transform *E. coli* HMS174 to ampicillin and tetracycline resistance (Ap^r and Tet^r, respectively). From two experiments, plasmid DNA from a total of 104 colonies was screened by *Hin*dIII and *Bam*HI/*Sma* I digestion and gel electrophoresis (data not shown). Four candidate plasmids that appeared to posses a complete AdBHG genome were selected and all four were found to be infectious when transfected into 293 cells (data not shown). Since large palindromes, like that created by head to tail joining of the ITRs in these clones, are not compatible with plasmid replication in most strains of *E. coli* and result in rearrangements or deletions that disrupt the palindrome structure (10, 28, 29), the ITR junctions in each of the infectious clones were sequenced and analyzed. The number of nucleotides missing from the midpoint of the palindrome in each clone varied from as few as 4 bp (1 bp from the right ITR and 3 bp from the left) to as many as 19 bp (1 bp from the right ITR and 18 bp from the left). Because plasmids containing long palindromes tend to be unstable, we chose the clone missing 19 bp from the junction for further work. This plasmid was called pBHG9.

The final step involved generation of pBHG10 by deleting the packaging signals in pBHG9 by partial *Bam*HI digestion and religation (Fig. 1). Screening for pBHG10 was facilitated by the fact that removing the packaging signals also deleted the Tet^r gene. pBHG10 contains Ad5 DNA sequences from bp 19 (left genomic end) to bp 188; bp 1339–28,133 and bp 30,818–35,934 (right genomic end). The left and right termini of the Ad5 genomes are covalently joined and a segment of plasmid pBR322 is present between Ad5 bp 188 and 1339 to allow propagation of pBHG10 in *E. coli*. A *Pac* I restriction enzyme site, unique in this plasmid, is present between Ad5 bp 28,133 and bp 30,818 to permit insertion of foreign genes. Because the packaging signal is deleted, pBHG10 is noninfectious but cotransfections with plasmids that contain the left-end Ad5 sequences including the packaging signal produce infectious viral vectors with an efficiency comparable to that obtained with pJM17 (20) (unpublished data and see below).

Additional Alterations to pBHG10: Vectors with wt E3 Sequences or with an Expanded Deletion in E3. The use of plasmids such as pBHG10 allows for rapid and relatively simple manipulation of the Ad genome. Two modifications of pBHG10 are described in this section. Since for some applications it may be desirable to generate Ad vectors with intact wt Ad5 E3 sequences, we reintroduced wt E3 sequences into pBHG10 (Fig. 2). First, pBHG10 was digested with *Spe* I, which cuts only at 75.4 mu in Ad5 sequences, and ligated with pFG23K also linearized with *Spe* I, generating pBHG10A that now contains the desired wt E3 sequences in tandem with the previous E3 region containing the 2.69-kb deletion. To remove repeated sequences, pBHG10A was partially digested with *Nde* I and religated, generating pBHG10B. The kanamycin-resistance (Kn^r) segment was removed from pBHG10B by partial *Xba* I digestion and religation, generating pBHGE3. Except for the presence of a wt E3 region, pBHGE3 is identical to pBHG10 and is equally efficient for generation of Ad vectors with E1 substitution by cotransfection (unpublished).

For some applications, it may be desirable to have as large a deletion as possible within the E3 region. By utilizing a PCR and following a strategy similar to that described above for the construction of pBHGE3 (Fig. 2), we created a 3.13-kb E3 deletion and introduced it into pBHG10. The resulting plasmid pBHG11 is identical to pBHG10 except for an expanded E3 deletion that removes sequences from bp 27,865 to 30,995. Like pBHG10, pBHG11 contains a unique *Pac* I restriction enzyme site in place of the deleted E3 sequences to permit insertion of foreign genes. A detailed description of the construction of pBHG11 is available on request from the authors.

Construction of E1 Deletion Plasmids for Use in Cotransfections with pBHG Vectors. Plasmids pBHG10, pBHG11, and pBHGE3 were designed to contain all the essential Ad5 sequences required to produce infectious virus upon transfection of 293 cells except for the packaging signal (194–358 bp) needed to encapsidate viral DNA into viral particles. To generate infectious viral vectors, these plasmids or derivatives with an insert in E3 must be cotransfected into 293 cells with a second plasmid containing left end viral sequences including the packaging signal as illustrated in Fig. 3. To maximize the capacity of the BHG vector system, we required a plasmid with the largest possible E1 deletion for cotransfections with the BHG plasmids. Analysis of E1 sequences revealed that 3.2 kb could be deleted between an *Ssp* I site at 339 bp and an *Afl* II site at 3533 bp (Fig. 4). This

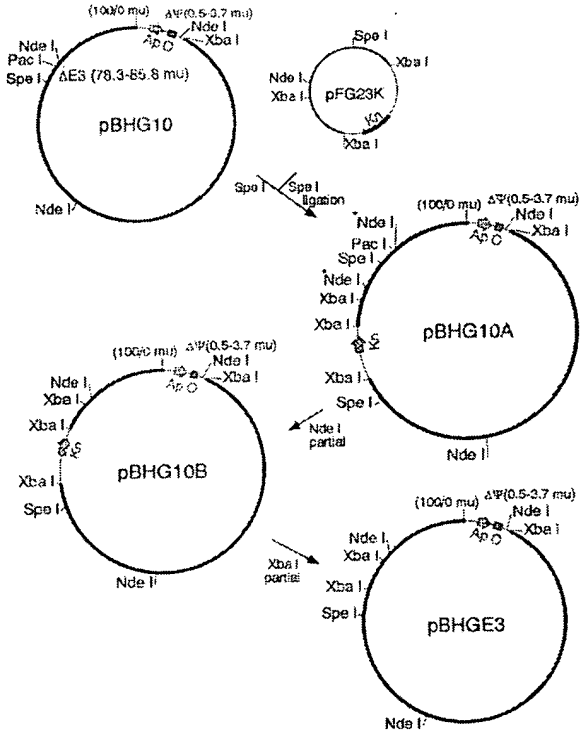

FIG. 2. Construction of pBHGE3. For insertion of wt E3 sequences, pBHG10 was digested with *Spe* I, which cuts at 75.4 mu in Ad5 sequences, and ligated with *Spe* I-digested pFG23K, generating pBHG10A. pFG23K was derived from the Ap^r plasmid pFG23 (30) (not shown), which contains Ad5 sequences from 60 to 100 mu. pFG23 was digested with *Xba* I, which cuts at bp 28,592 in Ad5 sequences (there is no cleavage at bp 30,470 due to Dam methylation in the *E. coli* strain used), and ligated with *Xba* I-digested pKN30 (31), a small Kn^r plasmid, generating pFG23AK (not shown). To remove Ad5 sequences that were not required and the Ap^r gene, pFG23AK was digested with *Afl* II and ligated, generating pFG23K. To remove the repeated sequences (shown between the asterisks), pBHG10A was partially digested with *Nde* I and religated, generating pBHG10B. In the final step, the Kn^r segment was removed from pBHG10B by partial *Xba* I digestion and religation, generating pBHGE3. mu refers to Ad5 sequences, solid bars represent Ad5 sequences, and hatched bars represent Ap^r and Kn^r segments.

deletion does not interfere with the ITR (1–103 bp), the essential core packaging signal (194–358 bp) (3–5), or coding sequences for protein IX, but does remove the Sp1 binding site (3525–3530 bp) from the protein IX promoter. Since the Sp1 binding site is thought to be essential for protein IX expression (32), it was reintroduced as a synthetic oligonucleotide that positioned the Sp1 site 1 bp closer to the protein IX TATA box (Fig. 4). To assess the effect of the 3.2-kb E1 deletion and the reintroduction of the Sp1 binding site on protein IX expression, 293 cells were infected at 10 plaque-forming units per cell with a number of different viruses. These include viruses with no deletion in E1 (wt Ad5), one with a 2.3-kb deletion extending into the protein IX gene (dl313) (33), one with the 3.2-kb deletion described above (dl70-3), and viruses with the 3.2-kb deletion containing the human cytomagalovirus (HCMV) immediate early promoter (AdHCMV) or the human β-actin promoter (AdβAct) in the E1-antiparallel orientation with or without the reintroduced Sp1 binding site. After labeling with [^{35}S]methionine, cell extracts were harvested and samples were immunoprecipitated with anti-Ad2 protein IX antibodies and analyzed by SDS/PAGE. The results (Fig. 5) indicate that variable levels

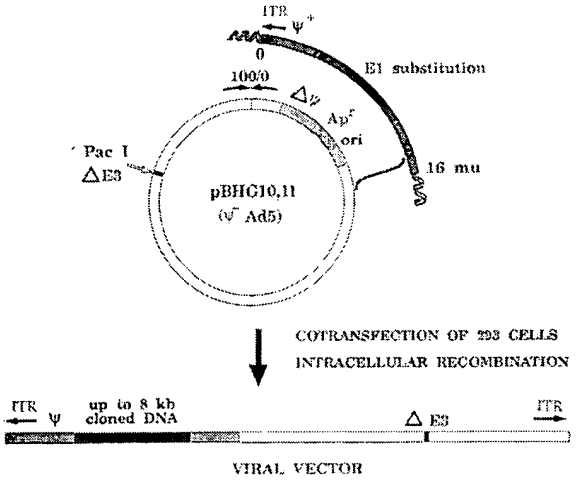

FIG. 3. Rescue using pBHG vectors. The general strategy used for generating infectious viral vectors using the BHG system is illustrated. Cotransfection of 293 cells with pBHG10, pBHG11, pBHGE3, or a pBHG derivative containing a foreign gene inserted in E3 plus a plasmid containing left-end viral sequences including the packaging signals results in the generation of infectious viral vectors by *in vivo* recombination.

of protein IX were expressed depending on the sequences upstream from the protein IX gene. With the reintroduced Sp1 site present, there was at most a 25% reduction compared to wt Ad5. The near-wt levels of protein IX expression obtained with mutant dl70-3 may be explained by sequences from left of the deletion (nt 333–338: GCGCGT, in Ad5 sequences) that fortuitously resemble and may act as an Sp1 site. The detection of reduced but significant levels of protein IX in cells infected by vectors containing only the HCMV or β-actin promoters, which have no potential Sp1 binding sites, suggests that the Sp1 binding site may not be absolutely essential for protein IX expression, in contrast to the findings of Babiss and Vales (32). Because protein IX is known to affect the heat stability of virions, we measured the infectious titers of wt Ad5 compared to dl313, dl70-3, AdHCMV2, AdβAct2, AdHCMVsp1, and AdβActsp1 after incubation at 45°C for 1 and 2 h. Of the six viral mutants tested, only dl313 differed significantly in heat lability from wt (Fig. 6). Even AdβAct2, which produces only 16% of wt levels of protein IX (Fig. 5), was as resistant to heat inactivation as was wt virus, suggesting that protein IX is likely made in excess during wt viral infection. We have also found that viruses containing the 3.2-kb E1 deletion replicate in 293 cells to the same final titers as wt Ad5 (data not shown).

Since the growth characteristics and stability of viruses with the 3.2-kb E1 deletion were not affected, this deletion was incorporated into pΔE1sp1A and pΔE1sp1B for use in cotransfections with the BHG plasmids (Fig. 4).

Testing the Efficiency and Capacity of the pBHG Vectors. To assess the ability of the BHG plasmids to generate infectious viral vectors, cotransfections with various left end plasmids were performed, and the efficiency of rescue was usually comparable to that obtained with pJM17 (20) (data not shown). Although pJM17 has been useful for rescue of E1 mutations or substitutions into infectious virus, because it is derived from dl309 (33), it has neither a wt E3 region nor a useful E3 deletion. Thus pJM17 will be superseded by the pBHG series of plasmids for most Ad5 vector constructions.

Use of pBHGE3, pBHG10, or pBHG11 combined with the 3.2-kb deletion in E1 should permit rescue of inserts of ≈5.2, ≈7.9, and ≈8.3 kb, respectively, into viral vectors. To test the capacity of the BHG system, we constructed an insert of

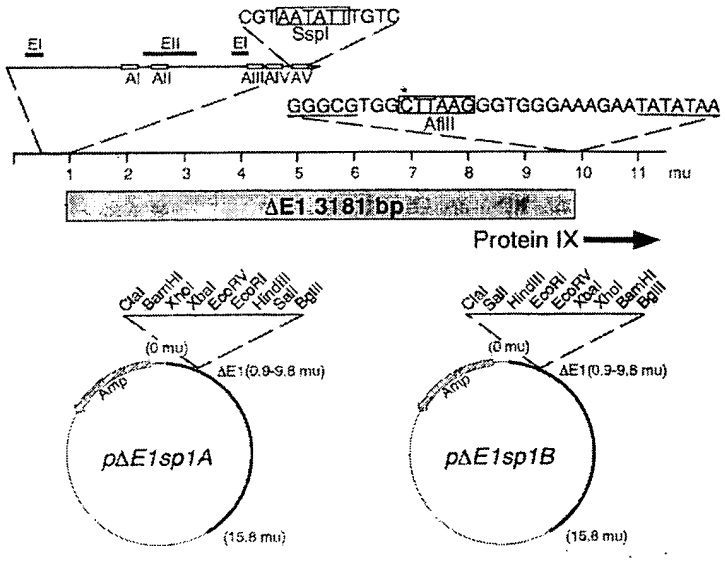

FIG. 4. Left-end shuttle plasmids with a 3.2-kb E1 deletion. The sequences removed by the 3.2-kb deletion are indicated. At the 5' end of the deletion the region from bp 190 to bp 348 has been expanded to show the position of repeated elements (AI–AV) involved in packaging and the enhancer elements (EI and EII). At the 3' end of the deletion, the region from bp 3525 to bp 3557 contains the protein IX promoter with the Sp1 binding site and TATA box (underlined). To create the E1 deletion, sequences were removed between the *Ssp* I (bp 339) and *Afl* II (bp 3533) restriction sites (boxed). This deletion does not interfere with the ITR (bp 1–103), the essential core packaging signal (bp 194–358), or coding sequences for protein IX, but does remove the Sp1 binding site (bp 3525–3530), which was subsequently reintroduced 1 bp closer to the protein IX TATA box. Thus the cytidine residue, indicated with the asterisk, is missing in plasmids pΔE1sp1A and pΔE1sp1B. In addition to the modified Sp1 site, plasmid pΔE1sp1A contains Ad5 DNA sequences from bp 1 to bp 341 and bp 3524 to bp 5790 with a polycloning oligonucleotide inserted between Ad5 bp 341 and bp 3524. Plasmid pΔE1sp1B is identical to pΔE1sp1A except that the restriction sites between the *Cla* I and *Bgl* II sites in the polycloning region are reversed.

7.8 kb consisting of the *lacZ* gene driven by the HCMV promoter (E1-antiparallel orientation) and the herpes simplex virus type 1 (HSV-1) *gB* gene driven by the simian virus 40 promoter (E1-parallel orientation) in the 3.2-kb E1 deletion. The 7.8-kb insert was constructed by inserting the 4.1-kb *Xba* I fragment from pgBdX17 (34) containing HSV-1 *gB* gene driven by the simian virus 40 promoter into the *Xba* I site in pHCMVsp1LacZ (35), generating pHlacZgBR (data not shown). After cotransfection of 20 60-mm dishes of 293 cells, 10 with 5 μg of pBHG10 and 5 μg of pHlacZgBR and the other half with 10 μg of each, one plaque was obtained. This plaque was isolated, expanded, analyzed by restriction digest with *Hin*dIII, and found to have the expected restriction pattern. The isolate, AdHlacZgBR, expressed both *lacZ* and HSV-1 *gB* at levels comparable to those obtained with vectors containing single inserts of these genes (data not shown).

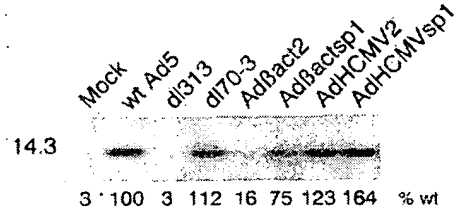

FIG. 5. Immunoprecipitation of protein IX from cells infected with viruses having a 3.2-kb E1 deletion. The levels of protein IX, a minor capsid protein required for the packaging of full-length viral genomes, were compared for wt Ad5 and viruses with a 3.2-kb E1 deletion with and without the reintroduced Sp1 binding site. The 293 cells were mock infected or infected at a multiplicity of infection of 10 with wt Ad5, dl313, dl70-3, AdβAct2, AdβActsp1, AdHCMV2, or AdHCMVsp1 and labeled with [^{35}S]methionine from 22 to 24 h after infection. Cell extracts were prepared and immunoprecipitated with anti-Ad2 protein IX antibodies, and samples were separated by SDS/PAGE. The gel was dried and bands were visualized by autoradiography. The lanes contain the samples indicated above and the molecular mass at 14.3 kDa is indicated on the left. Levels of protein IX were determined by densitometric analysis and are indicated below the samples relative to wt Ad5. dl313 contains a 2.3-kb deletion extending into the protein IX gene and, therefore, makes no protein IX; dl70-3 carries the 3.2-kb E1 deletion; AdβAct2 and AdHCMV2 contain the β-actin and HCMV promoters in the 3.2-kb E1 deletion, respectively; AdβActsp1 and AdHCMVsp1 contain the β-actin and HCMV promoters in the 3.2-kb E1 deletion with the Sp1 binding site reintroduced into the protein IX promoter. The β-actin and HCMV promoters were inserted in the E1-antiparallel orientation.

DISCUSSION

We have constructed and tested a vector system based on a series of bacterial plasmids (pBHG) that contain all the essential Ad5 sequences required to produce infectious virus upon transfection of 293 cells except for the packaging signal (bp 194–358) needed to encapsidate viral DNA. These plasmids are noninfectious in single transfections and must be cotransfected with a second plasmid containing left-end sequences including a packaging signal to generate infectious viral vectors (Fig. 3). This vector system is the most versatile system yet developed for generating Ad5 helper-independent vectors and will allow the construction of vectors with any combination of inserts, mutations, or wt sequences in both E1 and E3. Because with the pBHG system the entire viral genome is propagated as bacterial plasmids, manipulation of

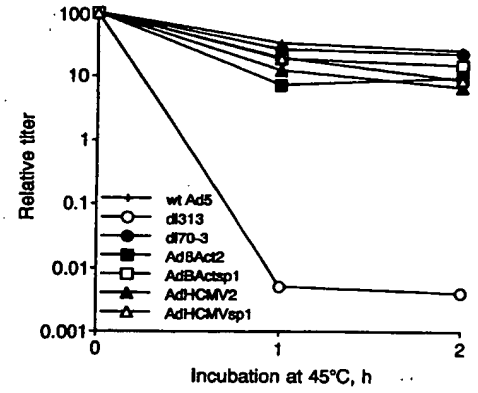

FIG. 6. Heat stability of viruses with the 3.2-kb E1 deletion. The heat stability of viruses with the 3.2-kb E1 deletion, with and without the reintroduced Sp1 binding site in the protein IX promoter, was compared to wt Ad5 and dl313. Viral stocks of wt Ad5, dl313, dl70-3, AdβAct2, AdHCMV2, AdβActsp1, and AdHCMVsp1 were titered on 293 cells prior to and after incubation for 1 and 2 h at 45°C. The structure of these viruses is explained in Fig. 5. The data presented for wt Ad5 and dl70-3 represent the average of four experiments and the data for dl313, AdβAct2, AdHCMV2, AdβActsp1, and AdHCMVsp1 represent the average of three experiments.

viral DNA sequences can be done rapidly and efficiently. To illustrate this and to generate two useful variants, we constructed pBHGE3 and pBHG11 from the original plasmid pBHG10. pBHGE3 permits construction of vectors with wt E3 sequences (Fig. 2), and pBHG11 increased the cloning capacity of resulting viral vectors. The 2.69-kb E3 deletion in pBHG10 removes the major portions of all E3 mRNAs, the first E3 3′ splice acceptor site, and the L4 polyadenylylation site but leaves the E3 promoter, the 5′ initiation site, the first E3 5′ splice donor site, and the E3b polyadenylylation site intact (36). Viruses with the 2.69-kb E3 deletion have the same growth kinetics and progeny virus yields as wt virus (7). The 3.1-kb E3 deletion in pBHG11 removes two additional elements not removed by the 2.69-kb E3 deletion: the first E3 5′ splice donor site and the E3b polyadenylylation site (36). This deletion does not interfere with the open reading frame for pVIII or any of the L5 family of mRNAs. Viruses containing the 3.1-kb deletion were found to give wt progeny yields in infected 293 cells (data not shown).

To maximize the capacity of the BHG system and to facilitate the introduction of inserts into the E1 region, we have constructed plasmids containing a 3.2-kb deletion of E1 sequences and multiple restriction sites for the insertion of foreign genes (Fig. 4*B*). This deletion leaves intact the left ITR and packaging signals and extends just past the Sp1 binding site of the protein IX promoter. The promoter for transcription of the protein IX gene is relatively simple, consisting of this Sp1 binding site and a TATA box. It has been reported that the Sp1 binding site is essential for expression of protein IX (32) and it was, therefore, reintroduced at a position 1 bp closer to the TATA box than in the wt promoter. However, neither the original 3.2-kb E1 deletion nor the deletion mutants containing the synthetic Sp1 site appeared to be significantly altered in protein IX expression (Fig. 5), heat stability (Fig. 6), or final progeny yields of viruses with this deletion. Although we did see a reduction in protein IX expression to ≈16% of wt levels for the virus containing the β-actin promoter combined with the 3.2-kb E1 deletion, even this level of expression appeared to be adequate for the formation of stable virions.

The Ad5 packaging signal, which overlaps the E1A enhancer region, has been found to consist of at least five AT-rich elements, which, by extensive mutational analysis, have been found to be functionally redundant (Fig. 4) (3–5). The E1A enhancer is composed of two functionally distinct enhancer elements, I and II (2, 37). Two repeats of enhancer element I flank element II and are responsible for regulating expression from the E1A gene (2). Enhancer element II regulates the transcription of all the early regions in the genome (37). The 3.2-kb E1 deletion does not interfere with the enhancer region but does remove the 3′ most packaging element. The removal of this element has been shown to have little or no effect on packaging (3–5) and should not, therefore, affect the packaging of recombinants that utilize the 3.2-kb E1 deletion.

One observation made when testing the BHG system was that the larger the insert being rescued in the E1 region the lower the efficiency of rescue. Although we have not systematically investigated the relationship between insert size and efficiency of recombination between cotransfected plasmids, we have observed that longer segments of foreign DNA seem to be more difficult to rescue into infectious virus than small inserts. This could be due to the inhibitory effect of heterologous sequences on recombination in agreement with the observations of Munz and Young (38).

When using pBHGE3, pBHG10, or pBHG11 in combination with the 3.2-kb deletion in E1, it should be possible to rescue inserts of up to 5.2, 7.9, and 8.3 kb, respectively, in conditional helper-independent vectors. To test the capacity of the system, we used pBHG10 to rescue a 7.8-kb insert consisting of the HSV-1 *gB* gene and *lacZ* gene in tandem, each with its own promoter. The vector obtained, AdHlacZgBR, was found to replicate efficiently and to express both *lacZ* and HSV-1 *gB* at levels comparable to that obtained with Ad vectors containing single inserts of these genes. The pBHG system has now been in use in our laboratory for ≈1 year and has facilitated the rescue of a variety of genes into E1 and E3. This vector system should have wide applications for the construction of Ad vectors for use as recombinant viral vaccines and for gene therapy transfer vectors.

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